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Identification of an immunogenic candidate for the elicitation of severe acute inflammatory reactions (SAIRs) to hylan G-F 20

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There have been numerous recent publications reporting an association of pseudoseptic reactions, or severe acute inflammatory reactions (SAIRs), with the use of hylan G-F 20 injections (Synvisc®, Genzyme Biosurgery, Cambridge, MA) as treatment for pain associated with osteoarthritis (OA) of the knee. These have recently been reviewed, and an additional four reports have appeared since that review[1–5]. The reactions are characterized clinically by 1) severe joint inflammation, 2) a typical requirement for more than one injection (e.g., after a second or third injection in a course or after initiation of a repeat treatment course), 3) the absence of infectious agents or calcium pyrophosphate crystals associated with sepsis or pseudogout, 4) high numbers of infiltrating mononuclear cells in the synovial fluid, and 5) the requirement for clinical intervention (e.g., arthrocentesis, intra-articular steroid injection, and/or non-steroidal anti-inflammatory drugs)[1]. To date, although there has been one report in the literature from a non-crosslinked fermented hyaluronan product[6], there have been no published accounts of these reactions occurring in patients receiving the avian-derived, naturally extracted sodium hyaluronate products approved in the US. Because the pseudoseptic reactions share characteristics with immune sensitization, it has been suggested that there may be a link between the pseudoseptic reactions and the chemical crosslinking process used to increase molecular weight (MW) during the manufacture of hylan G-F 20; this process is not used during the manufacture of currently marketed sodium hyaluronates[7,8]. We previously reported that seven subcutaneous immunizations of rabbits with hylan, but not sodium hyaluronate (MW 500–730 kDa, Hyalgan®, Fidia SpA, Padua, Italy), induced antibodies, as measured by enzyme-linked immunosorbent assay (ELISA), to the chicken proteins found abundantly in the source material for hyaluronan products. Rabbits immunized with a crude rooster comb preparation (CRC) were a positive control in the study[9]. Sasaki *et al.*[10] more recently reported that injections of animals with hylan, but not sodium hyaluronate (MW 620–1160 kDa, Supartz® or MW 500–730 kDa), induced specific antibody responses and granulomatous reactions, which may be related to the clinical reports of chronic granulomatous reactions in patients receiving hylan G-F 20[11]. Similar results have again recently been reported in guinea pigs and mice[12] and, in addition, animal data from Schiavinato *et al.*[13] suggest that a fulminant inflammatory reaction in the knee joint frequently occurs in response to hylan G-F 20 and not sodium hyaluronate after prior exposure to 1–2 intra-articular injections. Collectively, these studies suggest that these reactions may result from immune sensitization to a component of hylan that is not present in sodium hyaluronate preparations. The present study has extended previous findings by biochemically identifying a potential immunogenic protein present in hylan G-F 20 but not sodium hyaluronate (MW 500–730 kDa) that may serve as the target for the observed acute inflammatory reactions to hylan G-F 20.

A hyaluronidase-digested CRC preparation (undigested CRC ran as a smear) was separated on 4–20% sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) gels and blotted to nitrocellulose. Western blots of a CRC extract were then performed using the rabbit antisera from rabbits immunized with various sources of hyaluronans as previously described[9,14]. Bovine serum albumin-blocked blots were incubated with 1:500 dilutions of terminal bleed pools from each of the three groups of rabbits immunized with CRC, hylan G-F 20, or sodium hyaluronate (MW 500–730 kDa), respectively[9]. A quantitative ELISA using the same source of hyaluronidase-digested CRC preparation to coat ELISA plates was done to titrate the various lots of antisera (shown in Fig. 1). Immunoblots were then washed and developed using goat anti-rabbit horseradish peroxidase (1:2500) (KPL, Catalog #074-1516) followed by tetramethyl benzidine (TMB) substrate (KPL, Catalog #50-77-03). As shown in Fig. 2, anti-CRC antisera recognized multiple bands, but predominantly a 6- to 8-kDa protein band. Reactivity with a similarly migrating chicken protein band of 6 to 8 kDa was also seen with antisera from hylan-immunized rabbits. However, the sera from rabbits injected with sodium hyaluronate (MW 500–730 kDa) had no reactivity with any band.

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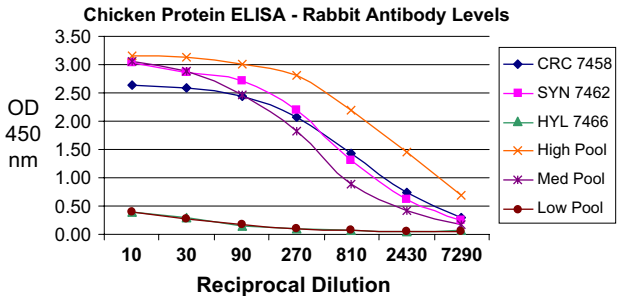

Fig. 1. Antibody titers from sera of rabbits immunized with various hyaluronan products used in Western blots. Rabbits were immunized with respective products a total of 7 times with approximately 2 mg of hyaluronan equivalents (CRC, Crude Rooster Comb preparation; SYN, Synvisc; HYL, Hyalgan), without adjuvant, over a 5-month period. Sera samples were collected at 6 months and individual rabbits, CRC Rabbit 7458, SYN Rabbit 7462, HYL Rabbit 7466, or pools of SYN immunized rabbits, High Titer Pool, Medium Titer Pool, and a Low Titer Pool, were analyzed at various concentrations. All samples were added to chicken protein coated, blocked and washed plates, and incubated for 1 h at 25°C. Detection of rabbit antisera occurred using goat anti-rabbit-HRP at 1:5000 in 1× PBS + Tween for 1 h, 25°C followed by TMB substrate for 10 min. The substrate reaction was stopped with 2 N H_2SO_4 and read at 450 nm. The High Titer Pool was used to detect protein bands in the Western blots for Synvisc.

Several lines of clinical evidence have implicated an immunologic basis for SAIRs. For example, reactions tend to require prior exposure or 'sensitization' and, in one clinical study, the incidence of SAIRs increased 10-fold with repeat vs single courses of hylan[15]. Additionally, Puttick *et al.*[16] found chicken-protein-reactive antibodies in serum from a patient with a SAIR. Several investigators have reported finding immune cell infiltrates in synovial fluid of patients despite the absence of infection, and bilateral reactions have been seen, suggesting in some cases there may be systemic involvement[1]. It has also been recently reported that patients who experienced a SAIR following injections with hylan G-F 20 could be subsequently treated with sodium hyaluronate (MW 500–730 kDa) injections, with no further adverse reaction[4,17]; this further supports the possible immunologic specificity of the reaction to hylan.

The results of this preclinical study confirm the presence of a specific immunogenic protein species in hylan G-F 20 preparation but not sodium hyaluronate (MW 500–730 kDa), and suggest that an immune reaction to a 6- to 8-kDa protein within a CRC preparation contributes to this immunogenicity. It is possible that this protein is present in the natural hyaluronan source and mildly immunogenic. However, the concentration of the protein in the CRC that elicited this antibody response vs that possibly present in either of the finished hyaluronan products is likely to be substantially greater. It is unlikely that this protein, in the concentrations present in finished products, is itself highly immunogenic, as immunization with the naturally extracted sodium hyaluronate failed to elicit an antibody response. We consider it more likely that a derivatized, more immunogenic variant of this protein, resulting from the aldehyde crosslinking in the hylan manufacturing process, is the primary candidate. Indeed, we suggest that this component may play a role in the induction of an immunological reaction that may lead to SAIRs or the chronic granulomatous reactions that have been associated with repeated hylan injections[11]. Our current work is aimed at determining whether antibodies reactive with the 6-kDa protein species are present in the synovial fluid and sera from patients who have experienced SAIRs.

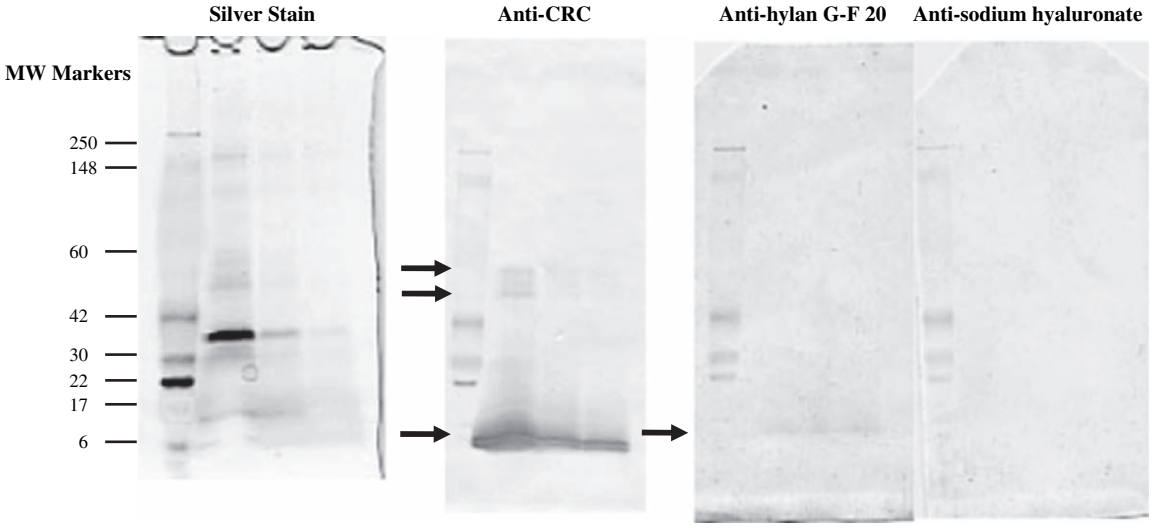

Fig. 2. Silver stain and Western blots of hyaluronidase-digested Crude Rooster Comb (CRC) (50, 12.5, and 7.5 μL per lane). Detection with anti-CRC rabbit sera blot shows reactivity with multiple bands in the 48- to 56-kDa region and a predominant diffuse band at 6- to 8-kDa protein. Reactivity with a 6- to 8-kDa protein is also observed almost exclusively with the anti-hylan G-F 20 rabbit sera blot. There is no reactivity observed in the anti-sodium hyaluronate rabbit sera blot.

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